Immune defense reactions of the desert locust, *Schistocerca gregaria* that survived infection by *Beauveria bassiana*

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Abstract: *Beauveria bassiana* is an entomopathogenic fungus that serves as a biological control agent of the desert locust, *Schistocerca gregaria* (F.) and other locusts and grasshopper pests. To measure the dose-dependent response to fungal attack, adult locusts were topically treated with doses of 4.5 x 10^4, 1.8 x 10^5, 4.5 x 10^5, 4.5 x 10^6 and 9.0 x 10^7 Bs/ul in sterile 0.85% saline with saline only as a control. LD_{30} was estimated to be 3.83×10^5 Bs/ul and used as sublethal dose to perform the subsequent immunological tests. Microscopic examination of haemolymph smears revealed that the fungal spores have been observed on the 1st day, and appeared darker on the 3rd day after infection. Haemolymph was taken from control and treated locusts, after different time intervals; 1, 2, 3 and 4 days post topical treatment, centrifuged and only the supernatant fractions were used for active phenoloxidase (PO), and lysozyme assays. A significant increase in PO was detected in fungal-treated insects at the 1st and the 2nd days post-treatment. This was followed by a significant decrease at the 3rd day compared with control insects. Also, a significant increase in lysozymes was observed at all time intervals post-treatment. We conclude that circulating PO and active lysozymes may be an important enzymatic defense against *Beauveria* infection and that they are associated with attempted clearing of *Beauveria* blastospores and hyphae from locust hemolymph.
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1. Introduction

The desert locust, *S. gregaria* (Forskal) is the most damaging pest amongst other locusts and grasshoppers (Lecoq, 2004). Due to the considerable drawbacks resulting from the use of chemical insecticides for controlling this pest, new approaches have directed toward the search for integrated locust control measures. Much attention has been devoted to use of entomopathogenic microorganisms. More recently, microbial control agents together with immunosuppressive mediators are required as part of a control strategy for this pest.

It is well known that insects lack an acquired immunity. Instead, they have highly developed innate mechanisms that comprise cellular and humoral reactions that cooperatively interact to destroy non-self elements (Gillespie *et al*., 2000). The cellular reactions are mediated by haemocytes, with the capacity to phagocytose and encapsulate invading microorganisms (Lackey, 1988). The humoral mechanisms involve the activation and release of substances found in the haemolymph. Some of these humoral factors may be derived from haemocytes and they have been reported to serve in defence as agglutinins, precipitins, lysins or opsonins (Engstrom, 1992). The insect humoral immune system has no immunoglobulin-like molecules to recognize foreign molecules. However, very little is presently understood concerning the molecular basis of non-self recognition by insects. A few candidate molecules such as lectins, haemolin, melanin, have been isolated from insects and may be involved in the recognition process as well as humoral factors (Engstrom, 1992). Recognition-associated molecules trigger signaling cascades, which activate immune system and induction of antimicrobial peptides that kill pathogens (Marmaras and Lampropoulou, 2009).

Humoral immune responses include activation of enzymic cascades that regulate coagulation and melanization of hemolymph, and production of reactive oxygen and nitrogen species (Mavrouli *et al*., 2005). Based on the substrate used to detect activity, these enzymes are commonly called monophenol and diphenol oxidases. Monophenol oxidases catalyze the hydroxylation of tyrosine to 3-(3,4-dihydroxyphenol)-L-alanine (L-Dopa) and diphenol oxidases catalyze the conversion of O-diphenols to the corresponding O-quinones (Nappi *et al*., 1987). Generally it has been accepted that these enzymes exist as catalytically inactive prophenoloxidases. These zymogens can be activated by lipases, proteases, zymosan, glucan and cell walls of fungi and bacteria (Söderhäll, 1982). Melanization is a complex enzymatic process in which melanin polymers cross-link with proteins (Hillyer *et al*., 2003). Consequently, phenoloxidase (intermediate product during melanization) is believed to be a key

mediator of immune function in insects, and has been implicated in non-self recognition and in resistance against various parasites and pathogens (Cotter and Wilson, 2002). For such reasons, the measurement of PO activity in the haemolymph has often to be used as an estimate of immune status and disease resistance.

In addition to the cellular defence reactions, many insects may be induced by the presence of certain elicitors to synthesize a range of antimicrobial substances (inducible proteins) in the plasma after infection (Gillespie *et al*., 1993). The most important one "lysozyme" is either present in the haemolymph of naïve insects or possibly released during haemocyte degranulation, hydrolyzes certain microbial components (peptidoglycan) produced a signal(s) which triggers fat body to synthesize antibacterial proteins (Jiang *et al*., 2011). Lysozymes are of several classes (Callewaert and Michiels, 2010) and generally exhibit greater antibacterial activity against Gram-positive than Gram-negative bacteria (Wang *et al*., 2009) as well as antifungal activity (Fiolka *et al*., 2005). Both haemocytes and fat body are reported to synthesize and release lysozymes into haemolymph (Lemaitre and Hoffmann, 2007). However, other tissues, such as epidermis, muscles and mid gut cells may also participate in lysozymes production (Hultmark, 1996).

There is little information on the inducible humoral components of the defense mechanisms in orthopteran insects. Therefore, the aim of the present study is to estimate the phenoloxidase and lysozyme titers in adults of S. *gregaria* that have successfully defended themselves against invasion of *Beauveria bassiana*. A better understanding of these mechanisms may provide us with baseline data about reasons for success or failure of fungal parasitism in insects, and might lead to new highlights in biocontrol of insect pests or at least, enable us to improve strategies of using mycopathogens in pest management.

2. Materials and Methods

Insects:

The desert locust, *S. gregaria* used in the present study was originated from Aswan (Upper Egypt) and established in the Locust and Grasshopper Research Department, Plant Protection Research Institute, Agricultural Research Center, Egypt. A colony from this locust was maintained in Faculty of Science, Ain Shams University and had shown no infectious diseases. The insects were reared in groups and maintained at 30 ± 2°C, 60 - 80% RH under a photoperiod of 16:8 (Light: Dark). Locusts were fed on an artificial diet as a dry mixture of: bran, 2; dried whole milk, 2; wheat, 2; dried brewer's yeast, 1 (parts by volume); plus a small quantity of fresh clover leaves. All experiments outlined below were carried out with adults (both sexes), all being within 2–4 days after ecdysis.

Tested fungi:

Commercial strain of *B. bassiana* (isolate GHA) obtained from Mycotech (Butte, MT) as a dry technical grade conidial powder was cultured on sabouraud dextrose yeast broth (SDY) at 26±2ºC in a rotator incubator for 2 days. Blastospores were then collected from filtrate by centrifugation and washed twice with sterile 0.85% saline as reported by Gillespie *et al.* (2000). A concentrated stock suspension in sterile 0.85% saline containing blastospores was prepared, and the blastospores were counted using pour plate method (Campbell and Konowalchuk, 1948).

***B. bassiana* dose response:**

Fungal treatments were topically applied to adult locusts at the posterior dorsum of the pronotum including the following fungal doses suspended in saline solution: $4.5x10^4$, $1.8x10^5$, $4.5x10^5$, $4.5x10^6$ and $9.0x10^7$ Bs/ul. An accurate delivery rate of inoculums was obtained using microapplicator equipped with a syringe according to Miranpuri and Khachatourians (1993). To avoid saprophytic growth of *B. bassiana* from the integument, cadavers were surface sterilized with 1% sodium hypochlorite for 3 min and then rinsed twice in sterile water. Surface sterilization ensured that fungal sporulation resulted from hyphae emerging from the body cavity, providing evidence of infection. Four groups of insects, each containing 15 individuals per dose were used in this assay. Control insects were treated only with equivalent amount of saline solution. Dead locusts were recorded according to a daily scheme, until nine days, in order to assess fungus-borne mortality rates.

One µl of fungal suspension (=LD_{30}) were prepared and used for investigating the humoral defense reactions.

Verifying infection:

10 μl of hemolymph was collected after 1, 2, 3 and 4 days from the coxal joint and directly smeared on a slide and stained with a drop of lactofuchsin. Ten insects were used for each test. Hemolymph samples were scanned at 400x, using light microscopy, for hyphae and blastospores.

Preparation of cell-free haemolymph: Haemolymph from normal and treated locusts after 1, 2, 3 and 4 days was drawn out as described above into Eppendorff tubes and then diluted five times with 0.85% saline solution. The haemolymph samples were then centrifuged at 2000 r.p.m. for 5 min, and only the supernatant fractions were used for assays directly. Ten replicates were used for each determination and the haemolymph of two individuals were never mixed.

***Phenoloxidase* assay:**

Phelonoxidase (PO) activity was determined spectrophoto-meterically by measuring formation of dopacrome in the plasma according to the method of Wilson *et al* (2001) based on Ashida and Soderhall (1984). 20 µl of plasma was added to 780 µl of 0.01 M ice-cold phosphate buffered saline (pH 7.4) and mixed in a plastic Eppendrof tube. Another 800 µl of 20 mM L-DOPA (Sigma) were added to each sample and the mixture was incubated at 25 ºC and the absorbance was read at 490 nm after 20 min. The phenoloxidase activity is expressed as PO unit per ml of plasma, where one unit is the amount of enzyme required to increase the absorbance by 0.001 min^{-1}. This test was replicated 10 times for each sample.

Lysozyme assay: enzymatic assay of lysozyme (EC 3.2.1.17) was followed according to Sigma instructions based on Shugar (1952). 1 ml of *Micrococcus lysodeikticus* (ATCC 4698) cell suspension (Sigma) in 66 mM potassium phosphate buffer, pH 6.24 (0.015%, w/v) was added to 40 µl lysozyme enzyme solution (containing 200 - 400 units/ml in cold phosphate buffer). Immediately the solutions were mixed by inversion, incubated at 25 ºC and the absorbance was read at 450 nm for approximately 5 min. Blank solution was with 40 µl of phosphate buffer. The decrease in absorbance was recorded. One unit was calculated as a ΔA_{450nm} of 0.001 per min at 25°C and pH 6.24 using a suspension of *M. lysodeikticus* as a substrate.

Statistical analysis:

To analyze the *B. bassiana* dose response data, we combined the data from both replicates because Fisher's Exact Tests indicated no significant differences between the replicates at each dose. The combined data were then subjected to probit analysis using LDP Line (LdP Line, 2000 by Ehab Mostofa Bakr, Cairo, Egypt). Other data were subjected to analysis of variance followed by Student-Newman-Keuls post-hoc tests (P = 0.05) using the ANOVA procedure of SAS (version 9.0). Mean values are presented with their standard errors (SE).

3. Results

Virulence measurement of the fungi:

In the control, mortality did not exceed 5%, and no sign of infection was observed on the dead locusts. After fungal treatment, mortality significantly increased four days and continued to increase to nine days after infection with an LD_{30} estimate of 3.83×10^5 blastospores/ul (Table 1).

Although the applied dose was lower, and more of the adult locusts survived the application, microscopic examination of haemolymph smears revealed that the fungal spores have been observed at the 1st day post treatment (Fig 1, A). Microscopic examination of the adult haemolymph smears also revealed that the formation of dense melanotic materials was observed in the adults three days post-infection. Brown material, probably melanin, appeared as a layer completely sealed the surface of the mycelia (Fig. 1, B). Melanization is considered as one of the related items to the phenoloxidase activity.

Phenoloxidase (PO) activity:

The mean value of the plasma PO activity in the haemolymph of untreated insects was 51.00 ± 8.56 unit/ml/min. Saline-treatments resulted in a significant decrease ($P\leq0.01$) in PO activity at the 1st and the 2nd days followed by a significant increase ($P\leq0.01$) thereafter compared with untreated insects. There were significant increases ($P\leq0.025$) in insects treated with *B. bassiana* at the first two days, followed with a significant decrease ($P\leq0.025$) at the 3rd day post-treatment compared with control insects (Fig. 2).

Lysozyme activity: Untreated insects had lysozyme activity of 101.00 ± 18.36 unit/ml/min. In saline-treated controls, there were significant increase ($P\leq0.025$) in lysozymes activities at all times post-treatment, compared with untreated insects. Also in *B. bassiana* treated adults, a linear significant increase ($P\geq0.025$) was detected at all time intervals post-treatment compared with the control insects (Fig. 3).

Table 1: Pathogenicity of *Beauveria bassiana* for adult *Schistocerca gregaria* based on mortalities nine days after injection

LD_{30} (blastospores/ul)	95% Confidence limits (blastospores/insect)	Slope (± S.E)	Chi-Square*	*g***
3.83×10^5	4.23×10^4 – 1.11×10^6	0.897 (± 0.162)	4.196	0.144

*Chi-square of heterogeneity: measures goodness of fit to the weighted regression line with $P > .05$ indicating a good fit of the data to the line.

**g is the index of regression significance.

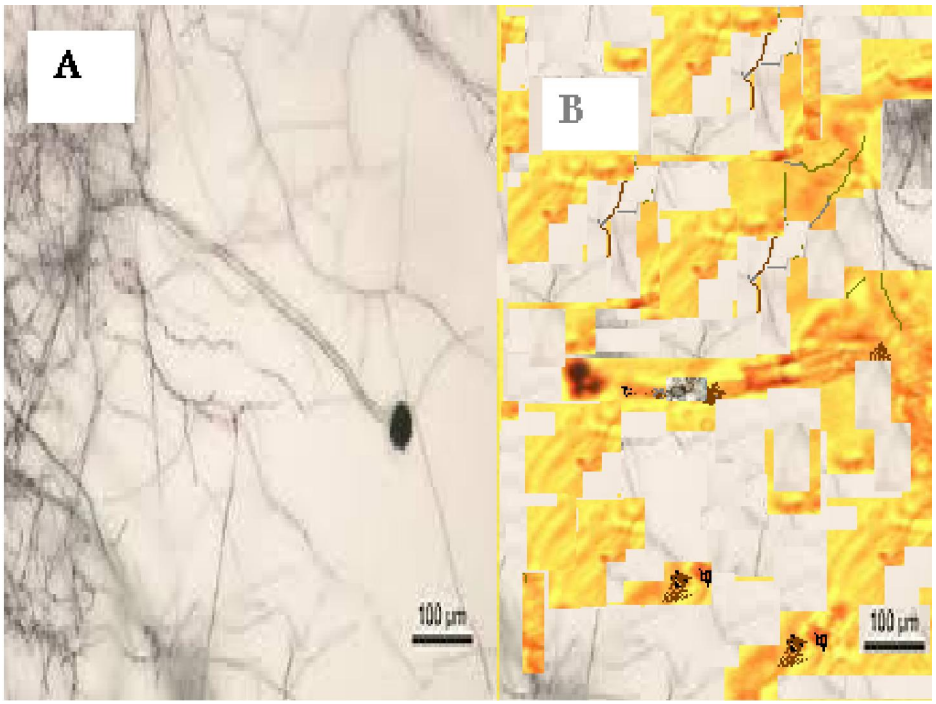

Fig. 1: Growing mycelia of *Beauveria bassiana* in the haemocoel of adult *Schistocerca gregaria* after topical application. (A) The 1st day post-treatment. (B) The 3rd day post-treatment (melanized). Bar = 100 µm.

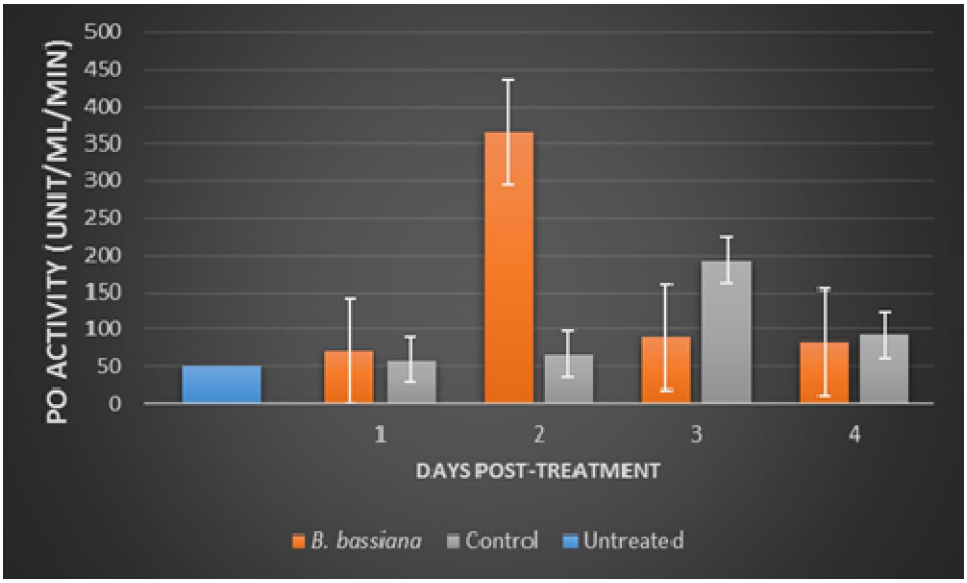

Fig. 2: Plasma phenoloxidase activity (PO) of adult *Schistocerca gregaria* determined at different time intervals post-topical treatment with saline (control) and *Beauveria bassiana*.

n = 10 replicates per test.

Comparison was carried out between the untreated and the controls and between the controls and the fungus-treated insects.

* Significant differences between the untreated and the controls when $P \leq 0.01$ and between the control and the fungus-treated insects when $P \leq 0.025$ based on Bonferoni correction.

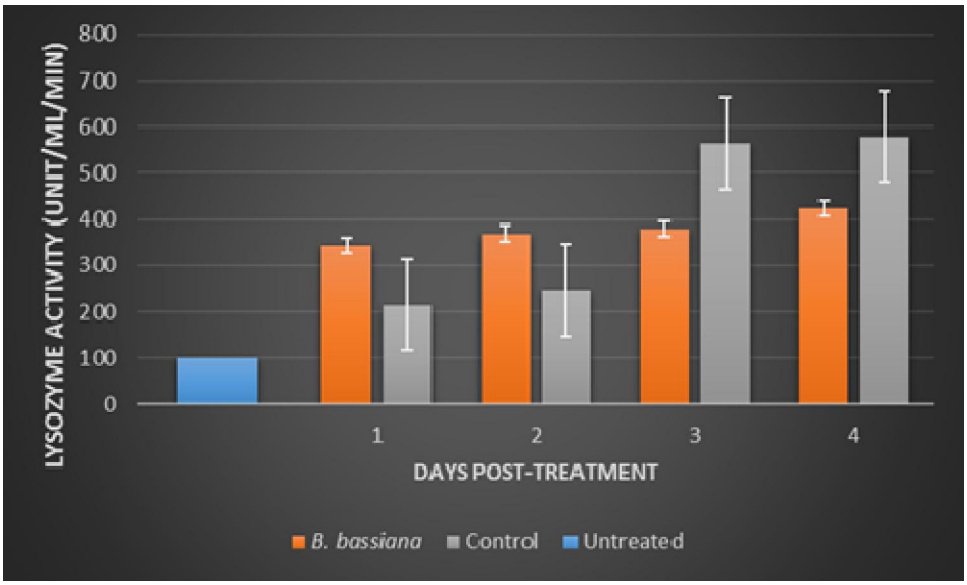

Fig. 2: Lysozyme activity in the plasma of adult *Schistocerca gregaria* determined at different time intervals post-topical treatment with saline (control) and *Beauveria bassiana*.
n = 10 replicates per test.
Comparison was carried out between the untreated and the controls and between the controls and the fungus-treated insects.
* Significant differences between the untreated and the controls when $P \leq 0.01$ and between the control and the fungus-treated insects when $P \leq 0.025$ based on Bonferoni correction.

Days post-treatment	**Lysozyme activity (Unit/ml/min) Mean ± SE**	
	Control	***B. bassiana***
1	215.00 ± 67.55	345.00 ± 135.32
2	246.50 ± 18.34	370.00 ± 73.74
3	380.00 ± 161.44	565.00 ± 111.24
4	425.00 ± 160.47	580.00 ± 246.60
Untreated	101.00 ± 18.36	

4. Discussion

The present study offers some new insights into the use of hyphomycete fungi as biocontrol agents for the desert locust, *S. gregaria* (Forskal). Within this frame, several questions should be answered. Are hyphomycete fungi suitable bio-control agents? and what are the interactions that take place within insect-body between the penetrating fungus and the immune system of the host? We evaluated for the first time the virulence of the hyphomycete fungous, *B. bassiana* against *S. gregaria*.

Lack of information on the quantity of test pathogen that reaches to the insect makes comparison of the infectivity of the pathogen, extremely difficult. This in turn would lead to difficulty in the standardization of insect pathogenic micro-organisms, and hence also in the evaluation of their potential in insect control. This factor has recently attracted considerable attention, to quantify the actual amount of pathogen introduced into the test insect.

In the present investigation, during virulence measurements, mortality of adult locusts began to increase significantly four days after treatment and tended to increase with time. Mortality may be attributed to the action of toxins secreted during growth of fungus within the insect-body which lead to malfunction of vital physiological activities. Additionally, depletion of nutrients due to mycosis and cessation of feeding causes eventually death (Mohamed *et al*., 1978). Variation in pathogenicity between natural infection and topical treatment of the hyphomycete fungi can be interpreted with that some fungi such as *B. bassiana* are more pathogenic on experimental infection than natural infection (Ignoffo *et al*., 1989). This phenomenon may be interpreted with the opinion of Boucias and Pendland (1984) that reported "the nutritional requirements of *B. bassiana* appear to be more fastidious than that reported for other entomopathogenic fungi".

The preliminary observations concerning verifying infection in the haemocoel of *B. bassiana*-treated insects revealed that melanotic capsules were detected. These observations support the findings of Goetz and Vey (1974) who reported that highly pathogenic fungi produce filaments able to break the capsular envelope, while the less pathogenic do not. Thus, these observations confirmed the concept that although humoral encapsulation may take place against invading hyphae of fungi, it does not prevent its colonization (Brey *et al*., 1988).

Humoral responses against parasitic fungi are poorly understood, and only a few antifungal peptides have been recognized (Vilcinskas and Götz, 1999). These responses need to be measured to evaluate the virulence of the tested entomopathogens. In insects, PO activity is an important part of innate immune system. It is a key enzyme in cellular and humoral immune responses as well as its active role as recognition factor of non-self molecules (Mavrouli *et al*., 2005). For such reasons, the measurement of haemolymph PO is used as an estimate of immune status and disease resistance.

In the present investigation, adult locusts treated with *B. bassiana* had greater PO activity above controls; results have shown that the PO activity increased significantly at the 1st and the 2nd days post-treatment. This increase indicates the activation of the immune system upon recognition of fungal material. *Beauveria* infection also increased PO levels in the grasshopper, *Melanoplus sanguinipes* (Gillespie and Khachatourians, 1992), the army cutworm, *Spodoptera exigua* (Hung and Boucias, 1996) and the cotton leafworm, *S. littoralis* (Meshrif *et al*., 2010). Gillespie and Khachatourians (1992) found that after topical application of 10^8 conidia to *M. sanguinipes*, PO levels increased 3.8 times peaking at 3 days post-infection. Results also showed, at the 3rd day, that PO activity decreased significantly and at the 4th day, it returned to near control levels. This late decrease may be attributed to the consumption of the PO in coagulation during cellular reactions (encapsulation and nodule formation) and further to the termination of antigenicity due to suppression of fungal infection. In contrast, our results contradict those of Bogus *et al*. (2007) that fungi significantly increased PO activity in *Calliphora vicina* that still unharmed, while in *Galleria mellonella* the PO activity dramatically dropped and eventually the insects died.

At the same time, Adamo (2004) reported that the ingredients of the immune response such as PO are not sufficient to find a relationship between the immune measuring and the actual resistance of the individual. These relationships are complex (Luster *et al*., 1993), for example, a measured decline in an immune response is not necessarily index on overall decrease in disease resistance. This decline could be an indicator of changing and redistribution of the immune components within the immune system to increase the immune strength against various invaders (Braude *et al*., 1999).

Additionally, lysozymes are potent means of the humoral defence components in insects, and considered to be the other partner of PO, which are able to estimate the strength of the immune system and disease resistance (Adamo, 2004).

Measuring of lysozyme activity in the pooled sample of haemolymph plasma is considered to be an accurate measure of its content in the whole insect body. This is because haemolymph is going on all parts of the body where they are synthesized and released. Results in the present seedy have shown that the constitutive level of lysozyme activity in the haemolymph plasma of naïve adult *S. gregaria* as about 101.00 ± 18.36. This is similar to that recorded in the hemolymph of several hemimetabolous insects, e.g., *Locusta migratoria* (Zachary and Hoffmann, 1984) and *Gryllus bimaculatus* (Schneider, 1985) and holometabolous insects, e.g., *Apis mellifera* (Mohrig and Messner, 1968), *G. mellonella* (Fiolka, 2012), *S. eridania* (Anderson and Cook, 1979), *Bombyx mori* (Morishima *et al*., 1995), and *Samiacynthia ricini* (Fujimoto *et al.,* 2001). This value is considered to be the background level of activity and the initial immune response against invading microorganisms. For instance, it is believed to degrade the bacterial debris released during the initial cellular immune response (Park *et al*., 1997).

Humoral immune responses, such as lysozymes and other antimicrobial peptides, which are synthesized and released into the haemolymph following microbial attack, are thought to be elicited by cell wall fragments resulting from the lysis of microbes (Kaneko *et al*., 2004; Park *et al*., 2007). This is thought to be a key-step in pattern recognition, which is followed by transduction of signals and initiation of immune responses (Tsakas and Marmaras, 2010).

Low levels of lysozyme activity were detected in untreated insect. As a result of this natural activity, insects can defend themselves against infection. Similar results were observed by Hultmark (1996) and Hoffman and Reichhart (2002) on *Drosophila*. It has been reported by Lavine *et al*. (2005) that lysozymes were found associated with some haemocytes, like granular cells, spherule cells and oenocytoids, and they were librating from these cells during lysis. Accordingly, the increase of lysozyme activity following fungal treatment can be attributed to haemocyte lysis. Moreover, these results confirmed the findings of Boucias *et al*., (1994) on *S. exigua* larvae, Meshrif *et al*. (2010) on *S. littoralis* larvae

challenged with *B. Bassiana* and Mohamed *et al.* (2013) on *S. gregaria* 5th instar nymphs challenged with different elicitors, where no marked changes in lysozymes were detected at all time intervals post-injection. These observations can be explained by two assumptions: (1) *B. bassiana* did not have the capability to induce lysozymes activation, and (2) they have been already under control of other arms of the immune system and therefore no need to activate lysozymes.

On the other hand, a significant decrease in lysozymes was reported in host-parasitoid interaction by Agsari and Schmidt (1994) and Agsari *et al.* (1997). This decrease could be attributed to the absence of antigenicity because the fungus may be able to avoid the induction of host lysozymes by coating themselves with a layer of soluble host proteins or by non-antigenic secretion. Additionally, at the end of the struggle between the fungus and haemocytes, the exhausted haemocytes are unable to recognize the non-self molecule anymore.

In a conclusion the circulating PO and active lysozymes of the desert locust, *S. gregaria* are quite effective as an important enzymatic defense against *Beauveria* infection and that they are associated with clearing of blastospores and hyphae from locust hemolymph. Moreover, as ingestion of nutrients, especially proteins, is associated with an increase of PO activity. Therefore, it is recommend more work to be done to understand the relation between metabolic pathways and immune system particularly with entomopathogenic fungi as promising bioinsecticides.

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